Review

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Transforming growth factor β latency: A mechanism of cytokine storage and signalling regulation in liver homeostasis and disease

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Summary

Transforming growth factor-β (TGF-β) is a potent effector in the liver, which is involved in a plethora of processes initiated upon liver injury. TGF-β affects parenchymal, non-parenchymal, and inflammatory cells in a highly context-dependent manner. Its bioavailability is critical for a fast response to various insults. In the liver – and probably in other organs – this is made possible by the deposition of a large portion of TGF-β in the extracellular matrix as an inactivated precursor form termed latent TGF-β (L-TGF-β). Several matrisomal proteins participate in matrix deposition, latent complex stabilisation, and activation of L-TGF-β. Extracellular matrix protein 1 (ECM1) was recently identified as a critical factor in maintaining the latency of deposited L-TGF-β in the healthy liver. Indeed, its depletion causes spontaneous TGF-β signalling activation with deleterious effects on liver architecture and function. This review article presents the current knowledge on intracellular L-TGF-β complex formation, secretion, matrix deposition, and activation and describes the proteins and processes involved. Further, we emphasise the therapeutic potential of toning down L-TGF-β activation in liver fibrosis and liver cancer.

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TGF-β signalling pathway

Transforming growth factor β (TGF-β) was identified as an inducer of anchorage-independent growth[1] and as the first member of the largest family of secreted morphogens in mammals. TGF-β family members are potent multifunctional cytokines that modulate many cellular processes, including cell differentiation and fate decisions of epithelial, mesenchymal, and inflammatory cell types; proliferation; extracellular matrix synthesis; production of inflammatory and fibrogenic cytokines/chemokines; tumorigenesis; and metastasis.[2] The TGF-β family, comprising at least 33 different members, is divided into distinct classes, based on biological function: TGF-β ligands, activins, inhibins, bone morphogenetic proteins (BMPs), nodal, Müllerian-inhibiting substance,[3,4] and growth differentiation factors (Fig. 1A). Perturbation of TGF-β family signalling may cause or facilitate various diseases, such as fibrosis, connective tissue, skeletal, cardiovascular, or autoimmune diseases, and finally, cancer.[4] Upon activation, the secreted protein ligands exert their cellular function via transmembrane type I and type II serine/threonine kinase receptors[5] and, canonically, intracellular Smad transcription factors.[6]

TGF-β family cellular signal transduction is principally mediated by a linear pathway including ligand binding to the type II receptor, recruitment of the type I receptor kinase, activation of signalling mediators (Smads),[7,8] translocation of Smad complexes into the nucleus, and regulation of target gene transcription.[9–11] Intracellular signal transduction by TGF-β family ligands is initiated by the binding and heteromeric formation of type II and type I receptors.[5,12,13] In many cases, the ligand-receptor complex includes another less described type III receptor, endoglin or TβRIII, that displays no kinase activity but facilitates ligand binding to the TGF-βR type II.[14] Receptor serine/threonine kinases currently comprise 12 members in the human genome – 7 type I and 5 type II receptors.[15] TGF-β ligand binding to its receptors at the plasma membrane may elicit multiple cellular responses.[5] Activated receptor complexes are usually internalised for signalling and subsequently undergo either proteasomal degradation or recycling to the cell surface.[16,17] These processes have a significant impact on the signal duration and the cellular response. Mechanistically, ligand binding induces a conformational change in the type II receptor, leading to autophosphorylation, recruitment of the respective receptor I kinase, and phosphorylation of the latter on a characteristic SGSGSG sequence of its GS domain.[5] This large active ligand-receptor complex initiates canonical signalling through phosphorylation of Smads.

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Smad proteins consist of three classes with distinct functions, the receptor-regulated Smads (R-Smads), the co-mediator Smads (Co-Smads), and the inhibitory Smads (I-Smads). Ligand-activated type I kinase receptors phosphorylate R-Smads (Smad1, 2, 3, 5, and 8) at C-terminal serines. Phosphorylated R-Smads form hetero-oligomers with Co-Smads (Smad4).[7] The activated heteromeric complexes then translocate into the nucleus, where they bind nuclear co-activators or co-repressors to direct chromatin decondensation and regulate target gene transcription in a context-dependent manner (Fig. 1B). Finally, the Smads may interact with a plethora of DNA-binding partners to initiate gene promoter activation or repression. Among the multiple potential target genes of Smad signalling are the I-Smads, Smad6 and Smad7.[18,19] Both form negative feedback loops, inhibiting R-Smad activation and mediating proteasomal degradation of activated receptor complexes by recruiting the E3 ubiquitin ligases Smad ubiquitin regulatory factors 1/2 (Smurf 1/2) to the TGF-β-receptor complex.[20] In addition, I-Smads interfere with R-Smad and Co-Smad interactions,[21] thus preventing transcriptionally active complex formation.

Besides the canonical Smad pathway, TGF-β family ligands may also directly affect other major intracellular signalling mediators, including JNK, p38, ERK, PI3K-AKT, TAK1-IKK-NFκB, and Rho family GTPases[22,23] (Fig. 1C). Activated by ligand-receptor complexes, non-Smad signalling transducers affect downstream cellular responses either by themselves or in crosstalk with the canonical Smad pathway.[24] Non-Smad pathways significantly contribute to the complexity of TGF-β effects, providing multiple options for intracellular signal branching and mutual interactions at regulatory nodes, resulting in a strongly context-dependent cellular response[22] (Fig. 1C-D).

Thus, although the TGF-β signalling pathway seems simple and linear, we know today that embedding the core components of the canonical TGF-β-Smad pathway within an extensive network of hierarchical protein-protein interactions leads to regulatory nodes at multiple levels that allow for substantial and highly context-dependent versatility.[16,25] For example, at the receptor level, accessory receptors such as TGF-βRIII and endoglin facilitate ligand binding. Furthermore, TGF-β receptor trafficking has also emerged as an essential regulator of Smad signalling,[26] indicating that the availability and activity of numerous components of the endosomal and trafficking system may have a critical impact. Finally, TGF-β signalling has cell type-specific outcomes. For example, TGF-β receptors modulate apical-basal polarity through Par6 and other components of the polarity complex in epithelial cell types, a feature that is disturbed during epithelial-to-mesenchymal transition (Fig. 1D). In fibroblasts, TGF-β drives cellular activation, in immune cells it is principally anti-inflammatory, whereas its effect on endothelial cells is pro-angiogenic.[27]

The present review will focus on the regulation of ligand bioavailability by intracellular and extracellular mechanisms at the TGF-β family protein level. We will describe the mechanisms and interactions that facilitate or interfere with the conversion of extracellular matrix (ECM)-deposited latent TGF-β (L-TGF-β) into an active signalling ligand (Tables 1 and 2).

Key points

- Transforming growth factor-β (TGF-β) signalling from the cell surface to the nucleus is regulated at multiple levels, starting from ligand activation, receptor complex formation, endocytosis, and recycling, via downstream pathway branching and co-factor recruitment, to target gene promoter binding.
- The pro-TGF-β dimer is cleaved into the TGF-β ligand and the latency-associated peptide (LAP) to form the small latent complex (SLC) via non-covalent interactions. The SLC is disulphide-bonded to the latent TGF-β binding protein (LTBP) to form the large latent complex (LLC). The LLC is secreted and stored as latent TGF-β (L-TGF-β) in the extracellular matrix via the LTBP.
- L-TGF-β activation is mediated by several signals, including integrins, thrombospondin, proteases, as well as compounds and chemicals which act through proteolysis or induction of structural alterations, all resulting in dissociation of the LAP.
- Extracellular matrix protein 1 (ECM1) protects L-TGF-β from integrin-mediated activation by competitively binding to the arginine-glycine-aspartic acid (RGD) motif present in most integrins.
- ECM1 depletion in the liver leads to robust spontaneous L-TGF-β activation, the release of the active ligand, and massive R-Smads activation in target cells, especially hepatic stellate cells, leading to progressive fibrosis and rapid mortality, indicating ECM1 as a gatekeeper for homeostasis in a healthy liver.

Intracellular formation and secretion of the latent TGF-β complex

TGF-β family mRNA is translated into pre-pro-TGF-β protein, which is transported into the endoplasmic reticulum (ER) after cleavage of the N-terminal signal peptide, where two pro-TGF-β polypeptide chains fold into a disulphide-bonded dimer.[28] Upon trafficking into the trans-Golgi, furin or furin-like proteases remove the mature TGF-β family ligand by proteolytic cleavage from the pro-polypeptide, which, however, remains non-covalently associated[29-32] (Fig. 2A). The dimeric complex consisting of the pro-peptides (latency-associated peptide [LAP]) and the TGF-β dimer is termed the small latent complex (SLC).[30,33] The non-covalent association of the components in the SLC involves extensive but reversible structural changes in the LAP.[34] Three cysteines in LAP are required for diverse functional interactions, Cys-223, Cys-225, and Cys-33. When transfected with a LAP plasmid encoding serine instead of cysteine at positions 223 and 225, cells express monomeric precursor proteins and release already activated TGF-β, suggesting that dimerisation of LAP is necessary for latency[35] and that Cys-223 and -225 are required for interchain disulphide bond formation.

The SLC in the ER is also covalently linked to the L-TGF-β binding protein (LTBP) via another pair of disulphide bonds, now between LTBP and LAP (Cys-33).[36-38] LTBPs are large multidomain glycoproteins that interact with fibrillin microfibrils in the ECM and build bridges with other matrix proteins, contributing to microfibril organisation and elastic fibre assembly.[36,39] There are 4 LTBP isoforms (LTBP-1, -2, -3, and -4) described in the human genome, with various affinities for LAPs.[40] LTBP-1 and LTBP-3 bind well to all three TGF-β isoforms; LTBP-4 binds only weakly to TGF-β1-LAP, whereas LTBP-2 does not bind at all. The tripartite complex of TGF-β, LAP, and LTBP is called large latent complex (LLC). LTBPs and LAP, thus play important roles in maintaining TGF-β latency.

LTBP isoforms are widely expressed in the normal and diseased human liver, as shown in liver specimens from patients with hepatitis B, hepatitis C, primary biliary cholangitis, and primary sclerosing cholangitis, and in cultured human liver myofibroblasts.[41,42] Upregulated expression levels of LTBP proteins (*e.g.*, LTBP-1) were described in cirrhosis and chronic hepatitis C.[43,44] Splice variants of LTBPs have also been described. LTBP-1D is less sensitive to proteolytic degradation, thus

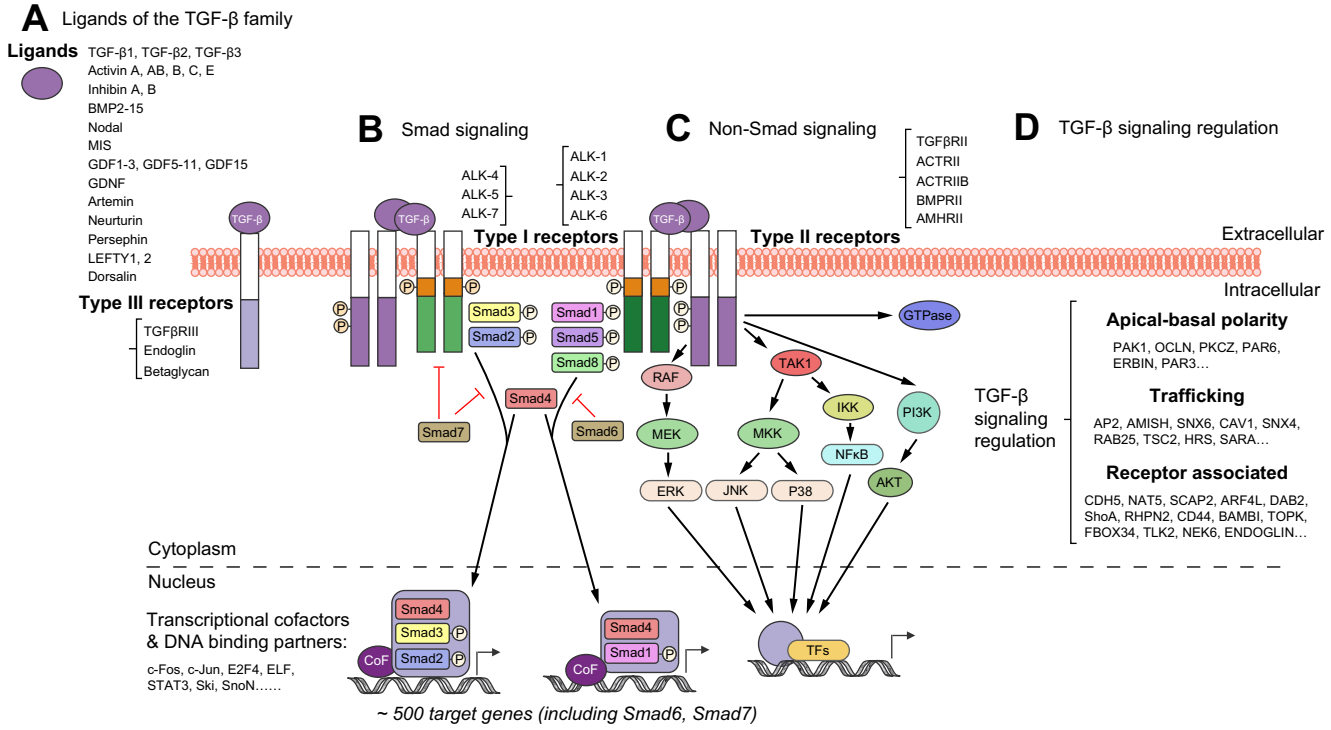

Fig. 1. The TGF-β signalling pathway. The scheme comprises different ligands and receptors of the TGF-β family, canonical Smad and non-canonical signalling pathways, and the network of regulatory interactions at multiple levels from the cell surface to the nucleus. (A) TGF-β family members, including TGF-β1, TGF-β2, TGF-β3, activins, inhibins, BMPs, nodal, MIS, and GDFs, initiate multiple cellular responses and cell fate decisions. (B) Secreted as protein ligands in latent and inactive forms, upon activation they may be presented by type III receptors (TGFβRIII or Endoglin) lacking kinase activity to the signalling receptor complex, consisting of heteromeric type II and type I receptors. Ligand binding to transmembrane type II receptors (*e.g.* TGFβRII, ACTRII, BMPRII) leads to autophosphorylation, recruitment and phosphorylation of type I receptor kinases (*e.g.* ALK-4, ALK-5, ALK-7 and ALK-1, ALK-2), thus forming a ligand-receptor complex to phosphorylate and activate the canonical Smad signalling pathway. Activated R-Smads (Smad2, Smad3 and Smad1, Smad5, Smad8, can be phosphorylated by ALK-4, ALK-5, ALK-7 and ALK-1, ALK-2, respectively) form hetero-oligomers with Co-Smads (Smad4), and the heteromeric complexes then translocate into the nucleus, where, in conjunction with variant co-factors and DNA-binding partners, such as c-Fos, c-Jun, ELF, or Ski, SnoN, and CEBP, they modulate chromatin decondensation and regulate target gene transcription. The I-Smads Smad6 and Smad7, which are direct transcriptional targets of Smad signalling, form crucial negative feedback loops to TGF-β signalling. (C) Non-Smad TGFβR signalling pathways are also frequently involved in regulating intracellular TGF-β signalling. TAK1 plays a central role, potentially coordinating the activation of MAPK signalling, including JNK, p38 MAPK, and NF-κB signalling. MEK-ERK, PI3K-AKT and some Rho family members like GTPases were also identified in the large TGF-β signalling crosstalk. (D) TGF-β signalling regulation occurs at multiple levels in extracellular and intracellular spaces. Thereby, the core components of the TGF-β-Smad signalling pathway are embedded within a huge network of hierarchical protein-protein interactions, leading to regulatory nodes at multiple levels. BMPs, bone morphogenetic proteins; Co-Smad, co-mediator Smad; I-Smad, inhibitory Smad; GDFs, growth differentiation factors; MIS, Müllerian-inhibiting substance; R-Smad, receptor-regulated Smad; TGF-β, transforming growth factor β.

protecting TGF-β from activation, and could be an important modulator of the biological activity of TGF-β in normal and diseased liver.[42] *Ltbp1* knockout (KO) mice exhibit reduced TGF-β activity and are less prone to hepatic fibrogenesis. Further, gene expression profiling of cultured hepatic stellate cells (HSCs) confirmed that *Ltbp1* KO cells are less receptive to cellular activation and less prone to transdifferentiation into myofibroblasts, which indicates that LTBP-1 has essential functions in TGF-β-

Table 1. Compounds, nucleic acids and peptides described as extracellular modulators of TGF-β signalling activation (in the order of appearance).

Molecules/compounds	Function	Ref.
MIR100HG	Long noncoding RNA, controls the magnitude of TGF-β signalling in carcinomas	59
GM6001	Metalloprotease inhibitor, abrogates αvβ8-mediated TGF-β activation in airway fibroblasts and SW480 cells	86,98
c8	Synthetic compound with inhibitory activity on αvβ1 integrin and downstream L-TGF-β activation in fibroblasts	103
CWHM12	Small molecule inhibitor of αv integrins, potential anti-fibrotic effects	105
LSKL	Peptide that blocks TSP-1-mediated TGF-β signalling activation	118
KRFK	TSP-1-derived TGF-β1 activating peptide	118
NDMA	Organic chemical, induction of hepatic fibrosis in mouse model	131
LAP-DP	Valuable biomarkers for monitoring L-TGF-β activation and the clinical course of chronic liver diseases	139
cRGD	Synthetic peptide blocking the interaction of αv integrin and L-TGF-β	182

LAP, latency-associated peptide; LAP-DP, LAP degradation product; L-TGF-β, latent TGF-β; NDMA, N-nitrosodimethylamine; TGF-β, transforming growth factor β; TSP-1, thrombospondin 1.

Table 2. Physiological proteins able to modulate extracellular TGF-β signalling activation (in the order of appearance).

Physiological proteins	Function	Ref.
Fibrillin-1	ECM structural protein, facilitates the deposition of L-TGF-β	63
Fibronectin	ECM structural protein, facilitates the deposition of L-TGF-β	73,74
Decorin	Storage of TGF-β in the ECM, decreases TGF-β activity	75,76
rhDecorin	Inhibition of TGF-β activity in HSCs	79
αv Integrins	Cell adhesion molecules, activate L-TGF-β and downstream signalling	85,88
Thrombospondin	ECM protein, activates L-TGF-β	114,116
ADAMTS	Facilitates the activation of L-TGF-β in HSCs	123
ADAMTS16	Activates L-TGF-β in cardiac fibroblasts	124
MMPs	Facilitate the activation of L-TGF-β	126,127,131
BMP1/tolloid MMPs	Regulate L-TGF-β activation via cleavage of LTBP	134
Kallikreins	Facilitate the activation of L-TGF-β	136,137
Prostate-specific antigen	Specifically activates L-TGF-β2 in prostate cells	140
Plasmin	Regulates the activation of L-TGF-β in co-cultures of pericytes and endothelial cells, in retinoid-treated endothelial cells, and on activated macrophages surfaces	141,142,145-147
ECM1	Inhibits αv integrin-mediated L-TGF-β activation	179,183
rECM1	Inhibitory effect on L-TGF-β activation in BMDCs and HSCs	179

ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; BMDCs, bone marrow-derived dendritic cells; BMP1, bone morphogenetic protein 1; ECM, extracellular matrix; ECM1, extracellular matrix protein 1; HSCs, hepatic stellate cells; L-TGF-β, latent TGF-β; MMPs, matrix metalloproteinases; rECM1, recombinant ECM1; rhDecorin, recombinant human Decorin; TGF-β, transforming growth factor β.

mediated HSC activation.[45] In hepatocellular carcinoma (HCC), mRNA analysis and immunostaining showed that while LTBP-1 was upregulated, LTBP-1D was not detectable in the matrix.[46] LTBP-1 in circulating plasma has thus been suggested as a novel biomarker for the early detection of HCC.[43] Aryl hydrocarbon receptor (AhR) signalling negatively regulates LTBP-1 expression.[47] Myofibroblasts from *Ahr* KO mice overexpress LTBP-1 and secrete 4-fold more active TGF-β than those from wild-type mice.[48] mRNA and protein levels of LTBP-2 are significantly upregulated in HCC tissues compared to adjacent

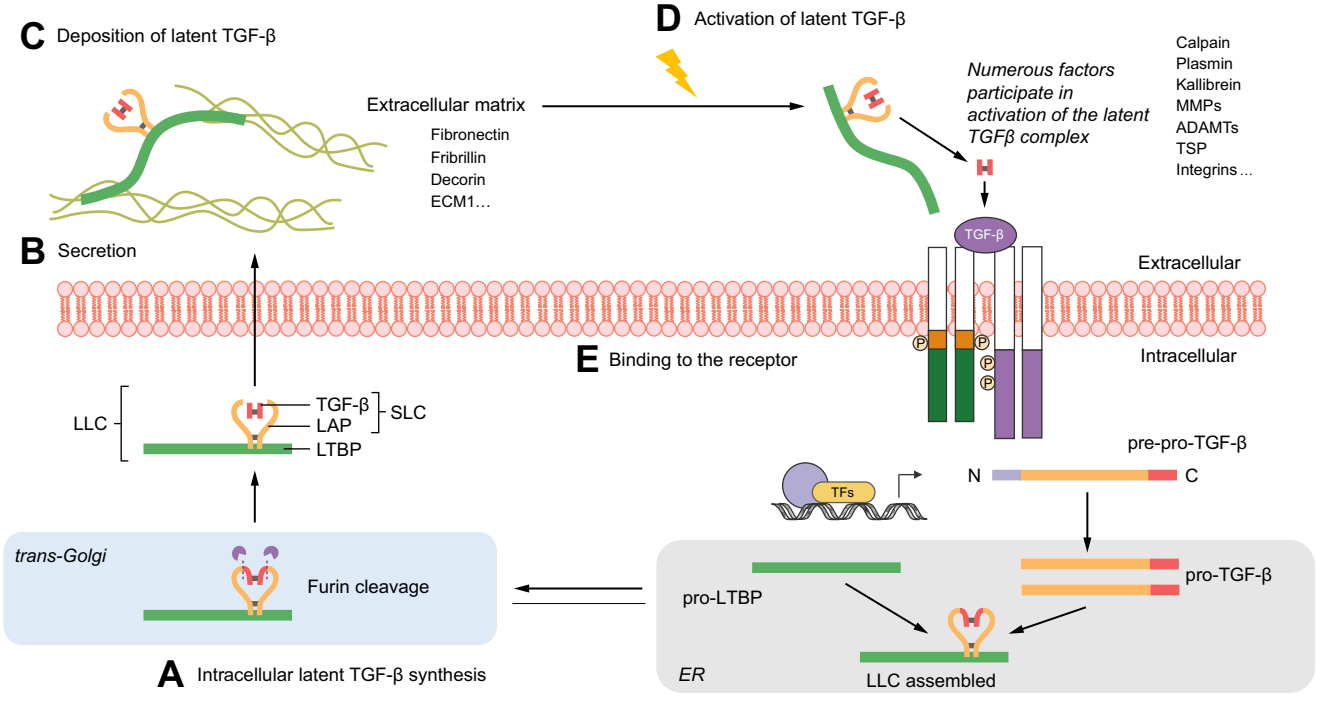

Fig. 2. Graphical summary of TGF-β protein production, secretion, matrix deposition, and maturation. (A) (starting at the bottom) After translation of pre-pro-TGF-β, the N-terminal signal peptide is cleaved to yield pro-TGF-β which translocates into the ER (lower right), where pro-TGF-β molecules dimerise and form disulphide bonds to LTBP, forming a ternary complex. The TGF-β dimer is cleaved from its pro-peptide in the trans-Golgi (lower left) but remains strongly associated with the LAP via non-covalent interactions. TGF-β and LAP form the SLC, which together with LTBP comprise the LLC. (B) Once secreted (middle left), the latent TGF-β is deposited into the extracellular matrix (C) (upper left), thanks to the high affinity of LTBP to various matrix components, such as fibronectin, fibrillin, and decorin. (D) (upper right) Numerous factors, like physiological proteins including proteases, thrombospondin and integrins, physical conditions and chemicals, including ROS and lactic acid, participate in activation of latent TGF-β, leading to the release of the mature TGF-β signalling ligand. The active ligand then binds to the cell surface receptors (E) (middle right) to regulate a wide variety of downstream signalling pathways and cellular responses.[28] ER, endoplasmic reticulum; LAP, latency-associated peptide; LLC, large latent complex; LTBP, latent TGF-β binding protein; ROS, reactive oxygen species; SLC, small latent complex; TGF-β, transforming growth factor β.

non-tumour tissue. Similarly, patients with HCC display higher serum LTBP-2 levels than controls, in line with the tumour's malignant degree, progression, differentiation, size, and stage, and with hepatitis virus infection, suggesting that LTBP-2 has prognostic implications in patients with HCC.[49,50] LTBP-4 exists as two N-terminally distinct isoforms, LTBP-4S (short) and LTBP-4L (long). The liver mainly expresses LTBP-4L. LTBP-4L is secreted as a complex with TGF-β1, whereas most LTBP-4S is free. Moreover, LTBP-4S is incorporated into the ECM, whereas LTBP-4L was not readily detectable in the ECM.[51] LTBP-4 is present in muscle and was originally identified from a genome-wide search for genetic modifiers of muscular dystrophy in mice. Two different alleles were found: one, containing a 12 amino acid insertion in the hinge region, had an enhanced affinity for L-TGF-β and led to reduced muscle fibrosis. LTBP-4 protein lacking those 12 amino acids is more susceptible to proteolysis and L-TGF-β activation, further supporting the important role of the hinge region. Moreover, a monoclonal human anti-LTBP-4 antibody directed towards the hinge region bound LTBP-4, inhibited proteolytic cleavage, and was beneficial in a mouse model of muscular dystrophy. Combining treatment with anti-LTBP-4 plus prednisone, the standard of care treatment for Duchenne muscular dystrophy, further improved muscle function and protected against injury in mice.[52]

TGF-β and its isoforms are principally secreted from cells in the latent and inactive form, either as SLC or LLC (Fig. 2B). After the cleavage process in the trans-Golgi, the LLC is prepared for secretion. LTBPs serve as chaperones to enhance the folding and secretion of pro-TGF-β.[53] While SLC secretion has sometimes been observed, it takes longer than for LLC due to the lack of LTBPs.[53,54] Without LTBPs, most of the SLC stays in the Golgi apparatus in a latent and immature form.[55] Tryptic digestion confirmed that without LTBPs, the precursor dimer contains improper disulphide bonding.[53] Furthermore, transgenic mice with a LAP carrying a substitution of Cys-33, the cysteine required for the interaction with LTBP, exhibit an inflammatory and tumorigenic phenotype similar to *Tgfb1* null mice.[56] The secretion of LTBP-3 also depends on binding to TGF-β, whereas LTBP-1, LTBP-2, and LTBP-4 can be secreted without linkage to TGF-β.[32,57] Secretion of L-TGF-β was also linked to autophagy since it was abrogated in fibroblasts and macrophages with depleted RHOA (autophagic component).[58] Additionally, a long noncoding RNA, MIR100HG, induced by TGF-β, facilitates L-TGF-β1 secretion and controls the magnitude of TGF-β signalling, especially in carcinomas.[59]

Deposition of the latent TGF-β complex in the extracellular matrix

The secreted LLC is rapidly deposited in the ECM via the association of LTBPs with ECM proteins.[60] Immunofluorescence studies revealed two ECM-binding regions of LTBP-1, one at the N-terminus and another at the C-terminus.[61] In addition, interaction studies showed that LTBPs bind to fibrillin fibres, fibronectin, and decorin (Fig. 2C).

Fibrillin-1 is the main ECM structural protein in humans and comprises 43 calcium-binding epidermal growth factor-like domains, 7 TGF-β binding protein-like domains, and an integrin-binding region.[62] The C-terminal domains of LTBP-1, LTBP-2, and LTBP-4 contain flexible linkers for binding to the N-terminus of fibrillin.[63] LTBP-3 does not interact with fibrillin.[63,64] In most cases, the assembly of the LLC with fibrillin fibres requires fibronectin, suggesting that a fibrillin/fibronectin network is responsible for the incorporation of LTBP/L-TGF-β into the ECM.[65,66]

Fibronectin (Fn) is an ECM glycoprotein that influences cell adhesion, migration, growth, and differentiation; it is particularly important in vertebrate development.[67-69] Fn is strongly expressed by multiple cell types and displays a profusion of interactions with collagens, integrins, fibrin, syndecans, and heparan sulphate proteoglycans, thus playing a crucial role in assembling complex fibrillar networks. Fn contains an arginine-glycine-aspartic acid (RGD) motif, the most common sequence required for cell attachment to the ECM,[70] which supports its central position in fibrillogenesis through binding to αv-class integrins.[69,71] Functional Fn is usually a dimer of 220 to 250 kDa subunits, covalently linked at their C-termini by a pair of disulphide bonds. Fn possesses two aggregated forms, the soluble plasma FN (pFn), and the insoluble cellular Fn (cFn). pFn is mainly synthesised in the liver by hepatocytes and circulates in the blood, while cFn is secreted by various cell types, including fibroblasts and HSCs.[72] Upon synthesis and secretion, cFn joins the ECM. Colocalisation of Fn and LTBP-1 was documented by double immunofluorescence.[73,74] Biochemical investigations indicate that Fn associates with a specific sequence of the LTBP-1 hinge domain, mediates localisation of the LLC into the ECM for storage, and is also required for subsequent activation by integrin αvβ6.

Decorin (DCN) is a small chondroitin-dermatan sulphate proteoglycan composed of a core protein with a single glycosaminoglycan chain near the N-terminus, 12 leucine-rich repeats, and N-glycosylation at three sites. The protein sequence comprises ten 24 amino acid repeats with a large proportion of leucine, also present in two other members of the DCN family, biglycan and fibromodulin. Decorin and biglycan do not bind L-TGF-β1, while all tested proteoglycans of the DCN family can bind the active forms of 3 TGF-β isoforms.[75] Collagenase treatment induces the release of biologically active TGF-β1/2 from collagen-bound decorin, indicating that decorin participates in the storage of TGF-β in the ECM.[76] Decorin decreases TGF-β activities in two ways, by sequestering the activated ligand into the ECM and by competitive inhibition of TGF-β binding to its receptors.[75] *In vivo,* DCN plays a protective role in liver fibrogenesis insofar as its genetic ablation in mice leads to enhanced matrix deposition, impaired matrix degradation, and activation of HSCs. TGF-β1 exerts a more potent effect when DCN is absent.[77] Blunting TGF-β1 in the *Decorin* KO setting is sufficient to reduce spontaneous collagen gel contraction activity, further confirming a regulatory interaction between decorin and TGF-β1.[78] Recombinant human decorin (rhDecorin) treatment inhibits the proliferation of TGF-β1-stimulated LX-2 cells, as well as the expression of metalloproteinase-2 (MMP-2), tissue inhibitor of metalloproteinase-1 (TIMP-1), alpha-smooth muscle actin (α-SMA), collagen type III, and Smad2 phosphorylation.[79] However, intrinsic expression of decorin along with TGF-β1 in liver tissue of 43 patients with chronic hepatitis and cirrhosis did not prevent liver fibrosis progression.[80] Overall, decorin is a highly specific natural inhibitor of TGF-β.[81]

Activation of TGF-β signalling ligands from ECM storage

As mentioned, TGF-β is present in a latent complex in the ECM until a specific signal triggers its release.[82] Several L-TGF-β regulators exist, including integrins, thrombospondin, proteases,

and other compounds and chemicals which act via proteolysis or by altering the binding and dissociation of LAP (Fig. 2D-E).

Integrins are transmembrane cell adhesion molecules. Heterodimeric receptors, consisting of α and β subunits on the outside of the cell surface, they link the ECM with the intracellular actin cytoskeleton and regulate specific signal transduction cascades.[83] Hence, they mediate communications between the ECM and cells, for instance, to trigger tissue fibrosis initiation, maintenance, and resolution. They possess the RGD tripeptide binding domain required for their interaction with L-TGF-β.[84-90] We currently know 24 integrin heterodimers. Eight of these, namely αvβ1, αvβ3, αvβ5, αvβ6, αvβ8, α5β1, α8β1, and αIIbβ3, can bind the RGD motif. Mice deficient in integrin subunit genes *Itgb3*, *Itgb5*, or *Itgb6*, and selectively deficient in *Itgb8* in haemopoietic cells are still sensitive to carbon tetrachloride (CCl_4)-induced experimental liver fibrosis in mice, whereas mice with myofibroblasts deficient in the *Itgav* gene are protected.[85] Exaggerated inflammatory diseases develop in the lungs and skin of mice with alterations in the integrin β6 subunit.[91] Inhibition of αvβ6 and αvβ8 reproduces the developmental phenotype of mice deficient in TGF-β1 and TGF-β3,[92] suggesting that these two integrins are required for most of these TGF-β isoforms' functions during development. Although, in principle, all αv integrins have the potential to activate L-TGF-β, mice deficient in αvβ3 or αvβ5 do not phenocopy TGF-β1 deficiency. Both are weaker binding integrins but may activate TGF-β1 signalling to some extent *in vitro*.[93,94]

L-TGF-β activation by αvβ6 integrins requires LAP's RGD motif and the integrin's cytoplasmic sequences that anchor the actin cytoskeleton. Upon secretion, cell surface-bound integrin provides cell contractile forces to the L-TGF-β complex via binding to the LAP's RGD motif. Since LTBP-1 – part of the complex – is attached to the stiff ECM, the binding of integrin creates counter traction deforming LAP, thereby facilitating the release of mature TGF-β.[95-97] This mechanism was experimentally confirmed by contracting myofibroblast cytoskeletons and integrins or anti-LAP antibody-coated ferromagnetic beads in a cell-free matrix.[96] The activation of L-TGF-β by αvβ8 is different from αvβ6. αvβ8 activates L-TGF-β by presenting LAP complexes to cell surface matrix metalloproteinases (MMPs) for degradation, subsequently releasing TGF-β. αvβ8-mediated activation can be blocked by an MMP inhibitor (GM6001) and does not need integrin's cytoplasmic domain, suggesting a traction-independent, probably protease-dependent mechanism involving the recruitment of MMPs to facilitate LAP cleavage.[86,98] The physiological relevance of integrin-mediated L-TGF-β activation was confirmed in mice, where an RGE sequence was used to replace the RGD motif in TGF-β1-LAP, resulting in a phenotype similar to *Tgfb1* KO mice with multiorgan inflammation and lack of epidermal Langerhans cells.[92,99] The integrin-binding RGD sequence is thus essential for both αvβ6 and αvβ8 integrin-mediated L-TGF-β activation.

L-TGF-β activation by integrins is an important cell fate decision mechanism during development and in response to TGF-β-driven organ diseases, especially fibrosis,[100] wherein depletion of integrins in mouse models attenuates disease severity in the liver, lungs, and kidneys.[85,101] Integrin αvβ6 mRNA is increased in patients with fibrotic liver disease secondary to various aetiologies (primary biliary cholangitis, alcohol-induced liver disease, hepatitis B and C), and a correlation with fibrosis stage has been documented for hepatitis C.[102] In contrast, no protective effect on liver fibrogenesis was found in the liver of αvβ6-deficient mice. c8, a synthetic compound with inhibitory activity on αvβ1 integrin, can interfere with the adhesion of TGF-β1 LAP to fibroblasts.[103] Systemic delivery of c8 reduces CCl_4-mediated liver fibrosis in mice.[89] Current knowledge on the role of integrins in the induction of TGF-β activity suggests that RGD binding could be a promising target for antifibrotic therapies. CWHM12, a small molecule integrin antagonist that simultaneously and selectively targets several profibrogenic integrins, improved liver, lung, muscle, and pancreatic fibrosis in mouse injury models.[104,105] Recently, hepatic steatosis, liver injury, inflammation, and fibrogenesis due to long-term high-fat diet feeding were decreased by CWHM12 treatment.[106] Extensive *in vitro* assays using a two-cell integrin-dependent TGF-β reporter system could discriminate L-TGF-β activation mechanisms in TGF-β1, 2, and 3, suggesting that the latent forms of TGF-β2 and TGF-β3 can be activated by integrin-independent mechanisms, which is not the case for TGF-β1. The presence of integrin further increased TGF-β3-dependent reporter activity by another 51%, which was blunted by mutating the RGD motif. Since TGF-β2 lacks an RGD motif, its activation is probably completely independent of integrin. Further, the furin cleavage sites conserved in all three TGF-β isoforms are required for integrin-dependent as well as for integrin-independent L-TGF-β activation. Finally, it has been shown that LAP1 more efficiently inhibits receptor binding of all three TGF-β isoforms than LAP2 or LAP3, suggesting that the respective LAP domains are involved in the observed intrinsic activities of TGF-β2 and TGF-β3.[107]

Thrombospondin (TSP) is a secreted matrisomal protein deposited in the ECM or bound to cellular receptors found in connective tissues and platelets. During tissue injury and repair its expression is rapidly induced by growth factors, including TGF-β, leading to the conversion of L-TGF-β to its biologically active mature form through a non-proteolytic mechanism.[108,109] This action, confirmed in different environments like in solution, at the cell surface, or in the extracellular milieu,[110] is relevant to diverse health conditions, including wound healing, immune responses, renal fibrosis, diabetes, myocardial infarction, tumour progression, and experimental autoimmune uveoretinitis.[111] TSPs are disulphide-linked homotrimers (subgroup A, comprising TSP-1 and TSP-2) or homopentamers (subgroup B, comprising TSP-3, TSP-4, TSP-5).[112,113] The interaction between TSPs and TGF-β is specific to TSP subgroup A members.[111] The WSHWSPW motif in the second thrombospondin type 1 repeat (TSR) of TSP-1 and TSP-2 can bind to VLAL motifs present in both TGF-β1 and the LAP,[114,115] thus localising inactive TGF-β1 at specific sites within the ECM or in proximity to cell surfaces.[111] Another interaction between a KRFK sequence of the second TSR and a conserved LSKL motif in the LAP can activate all three mammalian TGF-β isoforms by triggering their release from the SLC.[114,116] TSP-2 cannot activate TGF-β due to the lack of a KRFK motif but can competitively antagonise TSP-1-mediated TGF-β activation.[114,117]

Colony formation assays showed that TSP is similarly efficient in activating the large (platelet-derived) and small forms of L-TGF-β. Gel permeation chromatography suggested a direct binding of TSP to the LAP. Furthermore, a polyclonal antibody specific for the N-terminal region of the LAP inhibits TSP-mediated activation of L-TGF-β.[109] Young *Tgfb1* KO and *Tsp1* KO mice exhibit remarkably similar histological abnormalities in 9 organ systems, suggesting that TSP-1 is also largely responsible for TGF-β1 activation *in vivo*, in contrast to many other binding partners involved in storage and activation of L-TGF-β. Systemic

treatment with a peptide (LSKL) blocking the activation of TGF-β1 by TSP-1 is sufficient to induce pathologies in the lungs and pancreas that are similar to those observed in *Tgfb1* KO mice. In contrast, when using a TSP-1 peptide (KRFK) that activates TGF-β1, biologically active TGF-β can be detected *in situ*, and the abnormalities are reverted.[118] In addition, the TSP-1 derived from antigen-presenting cells that activates L-TGF-β2 is essential for the adaptive regulatory immune response induced by TGF-β2-expressing antigen-presenting cells and subsequent peripheral immune tolerance in TGF-β2-expressing tissues.[119]

TSP-1 is expressed in quiescent and activated cultured HSCs, and to a minor extent in Kupffer cells and liver sinusoidal endothelial cells in normal livers, but not in hepatocytes. Platelet-derived growth factor BB, a profibrogenic mediator, and to a minor extent TNFα, induce TSP-1 expression in HSCs, thus enhancing TSP-1-dependent TGF-β/Smad signalling. In HSCs, an LSKL peptide, specifically blunting the interaction between TSP-1 and L-TGF-β or antibodies against TSP-1, similarly inhibits the activation of L-TGF-β and active TGF-β's downstream effects.[120] Liver TSP-1 levels were increased in experimental obese and insulin-resistant NAFLD/NASH mouse models and in obese patients with NASH. *Tsp1* deletion in adipocytes does not protect mice from diet-induced NAFLD/NASH, whereas myeloid/macrophage-specific *Tsp1* deletion protects mice against liver injury. Autocrine-acting macrophage-derived TSP-1 suppresses SMPDL3B expression, thereby increasing Toll-like receptor 4-mediated proinflammatory signalling and promoting NAFLD progression.[121] Recently, BMP-1 was shown to be an activator of TSP-1 and an agonist of TGF-β signalling.[122] Besides acting as a vital regulator of TGF-β activation, TSP also has TGF-β independent biological functions, *e.g.*, on haemostasis, growth factor regulation, cell adhesion, migration, vascular barrier function, inflammation, inhibition of angiogenesis, and nitric oxide signalling through association with multiple integrins and other receptors.[111]

Several proteases belonging to different categories can activate L-TGF-β *in vitro*, including cysteine proteases like ADAMTS, and numerous serine proteases like MMPs, kallikreins, and plasmin. However, the relevance of these various proteases for L-TGF-β activation *in vivo* is unclear, as mice lacking individual proteases fail to show *Tgfb* KO phenotypes, perhaps due to functional redundancy.

A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) may activate L-TGF-β leading to increased expression of TGF-β target genes in HSCs, which themselves produce more ADAMTS1 when activated. ADAMTS1 expression is also upregulated in mice with CCl_4-induced liver fibrosis in parallel with type 1 collagen.[123] Another protease of this family, ADAMTS16, may activate L-TGF-β in cardiac fibroblasts via its RRFR-motif.[124]

Matrix metalloproteinases (MMPs) are calcium-dependent zinc-containing endopeptidases.[125] Despite documented activity on L-TGF-β, their actual physiological impact on TGFβ activation is not clear-cut. When presented to the tumour cell surfaces via CD44 during angiogenesis, MMP2 and MMP9 activate L-TGF-β.[126] MMP3 from chondrocyte-derived vesicles can activate small L-TGF-β1,[127] whereas MMP2, also present in the same vesicles, cannot. None of the many mice lacking these proteases clearly showed *Tgfb* KO phenotypes. MMP3 inactivation produces mice with no obvious abnormalities,[128] and mice lacking MMP13 develop only minor skeletal defects.[129] Mice lacking both MMP2 and MMP9 expression can survive,[130] like mice lacking MMP9 and MMP13, although these mice are very small because of the defects in their growth plates.[129] Levels of activated TGF-β and the fibrogenic markers connective tissue growth factor, α-SMA, type I collagen, and TIMP-1 are significantly decreased in *Mmp13* KO mice compared to WT mice following N-nitrosodimethylamine treatment.[131] Mice deficient in MMP14 display osteopenia, dwarfism, and fibrosis, but this is likely due to collagen turnover deficiencies rather than insufficient TGF-β activation.[132] MMP2- and MMP14-deficient mice suffer from respiratory failure, abnormal vascular development, immature muscle fibres, and die soon after birth.[133] However, it is difficult to match these phenotypes to defective TGF-β signalling. BMP1/tolloid MMPs also regulate L-TGF-β activation. Rather than degrading LAP directly, they promote the cleavage of LTBP-1 from the ECM, thereby freeing L-TGF-β bound to LTBP-1, which subsequently requires activation by other mediators, like MMPs. *Bmp1* KO mice with additional depletion of the closely related tolloid1 (Tll1) protease exhibit excessive deposition of LTBP-1 and reduced positive staining of phospho-Smad2/3 in embryonic tissues, suggesting a defect in L-TGF-β activation.[134] Mice deficient in both BMP1 and Tll1 expression die before birth.[135] However, their impacts on various other matrix and signalling molecules, and their importance for L-TGF-β activation *in vivo*, warrant further investigations.

Kallikreins can activate L-TGF-β, for example, in the context of TGF-β-mediated immunosuppression[136] and downstream of lipopolysaccharide during liver regeneration.[137] TGF-β1 LAP can be cleaved by plasma kallikrein between Arg-58 and Leu-59. The LAP degradation products (LAP-DP) are increased in liver tissue of patients with hepatic fibrosis.[138] Similarly, in patients with chronic liver diseases, Arg-58/LAP-DP were found around the sinusoids of fibrous regions (their levels were higher at fibrosis stage F1 than in more advanced stages). Similar observations in the plasma of patients suggest that plasma kallikrein-mediated TGF-β activation is an early step in liver fibrogenesis and that Arg-58 and Leu-59/LAP-DPs are valuable biomarkers for monitoring the clinical course of chronic liver diseases.[139] The authors also observed significantly higher expression of tissue LAP-DP in liver samples of from patients with NAFLD compared to patients with HCV-induced liver fibrosis, indicating a potential role of aetiology. Prostate-specific antigen, another kallikrein family member, may activate L-TGF-β2, but not L-TGF-β1.[140]

Plasmin regulates TGF-β activation in co-cultures of pericytes and endothelial cells,[141,142] an activation abrogated by antibodies against LTBP-1[143] and inhibitors of the mannose-6-phosphate receptor.[144] Similarly, plasmin activates L-TGF-β in cultures of retinoid-treated endothelial cells[145] and on the surface of activated macrophages.[146,147] The activation by macrophages depends on plasmin and L-TGF-β bound to the cell surface by TSP1 and CD36.[148]

Other binding proteins, including F-spondin,[149] neuropilin,[150,151] pregnancy-specific glycoprotein 1,[152] tenascin-X,[153] neuraminidase,[154,155] and cadherin-11[156] also, under certain conditions, have been identified as promoters of L-TGF-β activation, but more in-depth analyses are required. m-calpain's 80 kDa subunit, which requires chondroitinase ABC-sensitive proteoglycan for attachment to cell surfaces, may also activate L-TGF-β independently of plasmin.[157]

Activation by physical conditions and chemicals compounds

Exposure to specific physical or chemical conditions, such as detergents, chaotropic agents, ionising or UV radiation,[158,159]

reactive oxygen species (ROS),[160] heat,[161] physical shear stress,[162] and extreme pH[163] can activate L-TGF-β. For example, in cell culture and cell-free systems, ROS activate L-TGF-β efficiently.[160] ROS also seem to regulate L-TGF-β activation caused by ionising[160] and UV-B radiation.[159] Low pH-mediated L-TGF-β activation may play a physiological role in some circumstances. For example, extracellular pH is reduced to 4.5 by osteoclasts during bone resorption, which leads to L-TGF-β activation.[164] Lactic acid might promote TGF-β activity at specific concentrations and therefore has a physiological function in idiopathic pulmonary fibrosis.[165] Physical shear or stirring can facilitate the activation of L-TGF-β released by platelets, depending on LTBP binding to LAP.[162] Although there is convincing evidence that many physicochemical factors play essential roles in TGF-β activation, the detailed biological mechanisms are still unclear.

ECM1 – a new player in latent TGF-β activation

Extracellular matrix protein 1 (ECM1) is an 85-kDa glycoprotein[166] widely distributed in human tissues. It is present within the epidermis and the dermis,[167] where it maintains skin integrity and homeostasis.[168] ECM1 dysfunction may result in two skin diseases,[169] lipoid proteinosis (a rare autosomal recessive genodermatosis) and lichen sclerosus (a common acquired inflammatory skin disorder). Both have common clinicopathological features,[169] including skin hyperkeratosis, a hyaline appearance of the papillary dermis, and disruption of basement membranes. In addition, ECM1 can also regulate endochondral bone formation[170] or enhance endothelial cell proliferation.[171] Most ECM1 functions are mediated via interaction with various extracellular and structural proteins, such as perlecan,[172] fibulin-1C/D,[173] and MMPs (*e.g.*, MMP9).[174] Specifically, ECM1 interacts via its C-terminal domain with the epidermal growth factor-like modules flanking the LG2 subdomain of perlecan's domain V.[172] Disruption of this association seems to be responsible for some pathological features observed in lipoid proteinosis and lichen sclerosus.[172,175] ECM1's second tandem repeat domain interacts with the main interactive domain of fibulin-1D and the C-terminal module of fibulin-1C.[173] Fibulin-1D binding to ECM1 has been suggested to reduce endothelial cell proliferation and angiogenesis, thus reducing tumour transformation and invasion.[176,177] ECM1's C-terminal tandem repeat 2 interacts with MMP9, thereby inhibiting its proteolytic activity *in vitro*.[174] In addition, ECM1 also interacts with αv integrin, one of the aforementioned physiological regulators of L-TGF-β activation.[178] ECM1 inhibits differentiation of IL-17-producing T helper (Th17) cells and the development of experimental autoimmune encephalomyelitis through interference with αv integrin-mediated L-TGF-β activation,[179] suggesting clinical importance in multiple sclerosis. Furthermore, ECM1 levels are increased in most malignant epithelial tumours[180] and in tumours with metastases,[177] indicating ECM1 involvement during cancer development.

Most evidence of crosstalk between ECM1 and TGF-β came from immunology. In order to elucidate the communication between ECM1 and L-TGF-β, Su *et al.* tested *Tgfb* mRNA expression in bone marrow-derived dendritic cells (BMDCs) grown in the presence of recombinant ECM1 protein (rECM1).[179] *Tgfb* mRNA expression did not significantly change in BMDCs in the presence or absence of rECM1. However, when BMDCs in culture were treated with rECM1 protein and L-TGF-β, the level of active TGF-β in the culture supernatant was significantly reduced, as measured with a reporter cell line (HEK293T-cells stably transfected with a plasminogen activator inhibitor-1 (*PAI1*) promoter luciferase plasmid[181]). For the first time, these results indicated that ECM1 has an inhibitory effect on L-TGF-β activation. Th17 cell differentiation was induced during the co-culture of dendritic cells and $CD4^+$ T cells in the presence of IL-6, IL-23, IL-1β, and one of IgG, ECM1, or cyclic arginine-glycine-aspartic acid peptide (cRGD). TGF-β or L-TGF-β were then added to the co-culture system. The Th17 cell proportion – determined by intracellular cytokine staining for IL-17 and FACS analyses – was significantly reduced by adding rECM1 to the culture system in the presence of L-TGF-β but did not change in the presence of active TGF-β. rECM1 has a similar inhibitory effect to cRGD, which inhibits L-TGF-β activation by blocking the interaction of αv integrin and L-TGF-β.[182] Levels of active TGF-β in the supernatant decreased in the presence of rECM1, suggesting that ECM1 can suppress TGF-β maturation and Th17 cell differentiation as effectively as cRGD. Co-immunoprecipitation experiments identified a strong interaction between ECM1 and αv integrin, the latter being expressed on the surface of dendritic cells. When αv integrin was silenced in BMDCs, ECM1 could not inhibit Th17 differentiation in the co-culture system. Furthermore, in protein-protein interaction competition assays, ECM1 competed with the RGD peptide for αv integrin binding. ECM1 was thus identified as an inhibitor of L-TGF-β activation via binding to αv integrin, affecting TGF-β-driven Th17 cell differentiation.

We recently showed for the first time that ECM1 expression is a critical gatekeeper in the healthy liver, contributing to normal architecture and physiological homeostasis of cell-cell communication.[183] Hepatocyte-specific *Ecm1* KO leads to spontaneous and severe liver fibrosis. Via interaction with the αv integrin, ECM1 is critical for maintaining ECM-deposited TGF-β in its latent form (Fig. 3). Indeed, *Ecm1* KO mice develop severe liver damage and fibrosis and die at between 8 and 12 weeks of age. Real time-PCR results showed no significant differences in *Tgfb* mRNA levels between *Ecm1* KO and WT mice. However, in *Ecm1* KO mice, TGF-β signalling activation was dramatically induced, as evidenced by strongly upregulated levels of Smad3 phosphorylation. Immunoprecipitation and immunofluorescent staining confirmed that – like in inflammatory cells – ECM1, αv integrin, and L-TGF-β interact and co-localise in the hepatic sinusoids. We tested TGF-β activation *in vitro* in the different liver cell types isolated from *Ecm1* WT and KO mice. Co-culturing them with NIH3T3-TGF-β signalling reporter cells stably transfected with the *PAI1*-promoter-based luciferase plasmid, in the presence or absence of rECM1 or CWHM12 – an αv integrin inhibitor – showed that TGF-β is expressed and secreted primarily from HSCs and Kupffer cells, and that the amount of active TGF-β is much higher in cells isolated from *Ecm1* KO mice. As with CWHM12 treatment, the addition of rECM1 significantly reduced active TGF-β levels. These data suggest that ECM1 also affects L-TGF-β in the liver, which translates into HSC activation and progressive fibrosis. Significantly, injecting *Ecm1* KO mice with a vector expressing a soluble TGF-β type II receptor led to a decrease in collagen deposition, ACTA2-positive HSCs, and hydroxyproline levels and to a more or less normal liver architecture, rescuing the liver damage phenotype. Taken together, hepatic ECM1 depletion leads to spontaneous L-TGF-β activation and release of the active ligand, resulting in strongly enhanced TGF-β signalling, especially in HSCs, and is responsible for the onset of hepatic fibrosis in mice.

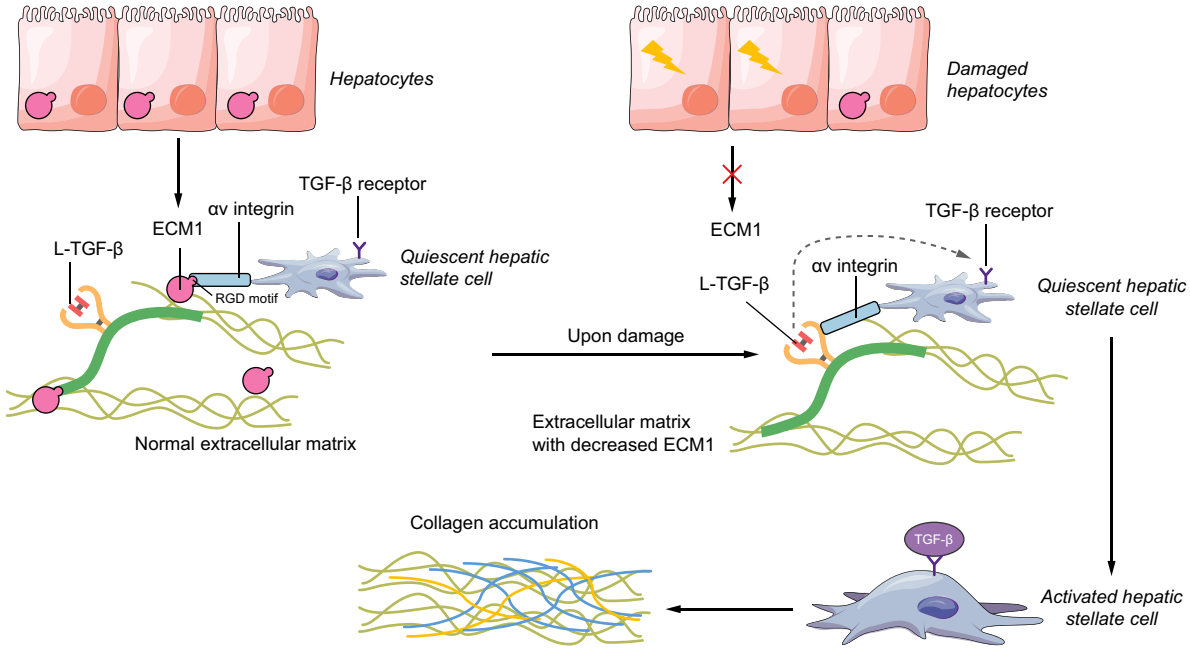

Fig. 3. Scheme showing how ECM1 sequesters the latent TGF-β from integrin activation. We recently found that ECM1, a glycoprotein in the extracellular matrix of the liver, is critical in maintaining normal architecture and physiological homeostasis. Under normal conditions, ECM1 binds to the RGD motif, which commonly exists in the LAP and most integrins, thus inhibiting the activation between integrins and latent TGF-β. The presence of ECM1 is critical to keep extracellular matrix-deposited TGF-β in a latent and quiescent form, preventing HSC activation and liver fibrosis. Conversely, depleting the liver of ECM1 results in spontaneous release of active TGF-β ligand from the latent complex, leading to strongly enhanced TGF-β signalling, HSC activation, collagen accumulation, ultimately promoting spontaneous architectural disturbances and severe liver fibrosis. ECM1, extracellular matrix protein 1; HSC, hepatic stellate cell; LAP, latency-associated peptide; L-TGF-β, latent TGF-β; RGD, arginine-glycine-aspartic acid; TGF-β, transforming growth factor β.

Therapeutic strategies targeting TGF-β signalling

The profibrogenic, anti-inflammatory, and pro-oncogenic impact of the TGF-β signalling pathway has encouraged the development of targeted therapeutic approaches for fibrosis and cancer. Tools have been generated to interfere with various levels of the TGF-β signalling cascade, including antisense oligonucleotides,[184,185] TGF-β ligand-targeting antibodies (*e.g.* fresolimumab[186] and 1D11[187]), ligand traps such as soluble TGF-β receptor II or AVID200,[188] as well as small molecule inhibitors directed to the internal part of the signalling receptors[27] or targeting Smad3 (SIS3).[189,190]

Results from preclinical studies targeting TGF-β in fibrosis or cancer have been very promising.[191,192] However, translating these approaches for clinical use has been and remains a challenge. Indeed, TGF-β signalling is critical for maintaining tissue homeostasis, and TGF-β displays pleiotropic effects. In addition to the "diseased" target cells, healthy tissues can be compromised by systemic or organ-wide delivery of TGF-β signalling-directed drugs, which can and frequently do lead to unwanted side effects and safety concerns.

To overcome these drawbacks and tap the full potential of TGF-β as a therapeutic target while increasing safety and efficacy, we need i) a better understanding of the molecular mechanisms by which TGF-β signalling controls homeostatic and disease processes, ii) predictive biomarkers from tissue and blood signatures to stratify patients that may benefit from TGF-β targeting,[193,194] iii) careful dosing or pulsatile treatment windows, and iv) a selection of potential targets from specific branches of TGF-β signalling pathways as is nicely summarised in the review article of ten Dijke and colleagues.[27] Targeting the mechanisms underpinning L-TGF-β activation is one such approach for a more fine-tuned interference with TGF-β. For example, EMD527040, a selective antibody against αvβ6 integrin, blocks the activation of L-TGF-β and inhibits fibrogenesis in bile duct ligated rats and *Mdr2* KO mice.[195] Similarly, antibodies against αvβ6 or αvβ8 integrin also display anti-tumour activity in preclinical mouse models through suppression of TGF-β signalling,[196,197] and clinical translation for patients with cancer is currently envisaged. Small molecules to target L-TGF-β integrin interactions are also available. For example, the cyclic RGD pentapeptide cilengitide (EMD121974) was efficiently used in several preclinical models of solid tumours[198] and is now being tested in clinical studies in patients with cancer, as summarised in ten Dijke's review.[27] Furthermore, the small molecule CWHM12 pharmacologically blocks αv-containing integrins, thus attenuating fibrosis in the liver and lung.[85] We hypothesise that delivery of ECM1 protein to the chronically damaged liver may be another promising approach that can delay the process of disease progression without major side effects.

Outlook

The bioavailability of TGF-β and TGF-β family members is just one of the multiple levels of regulation to fine-tune initiation, strength, duration, branching, crosstalk, and target gene regulation of TGF-β family signalling. Multiple major components can modulate TGF-β signalling in complex and diverse ways, as indicated in the Snapshot on TGF-β signalling regulation by Wrana and Taylor.[25] This complexity is the primary reason for the highly context-dependent impacts of activated TGF-β

signalling on cell types and the difficulties of developing specific therapeutics that do not induce unmanageable side effects. Our current knowledge on TGF-β bioavailability shows that, at least in the liver, a significant amount of L-TGF-β is deposited in the ECM and can be activated immediately without the need for *de novo* synthesis. We can assume that this will also be true for other tissues, although that must be proven. Thus, mRNA measurements might, in many cases, reflect a second wave of pathophysiological responses, *e.g.*, to replenish the emptied ECM deposits. It also seems that such an available reservoir of L-TGF-β is required for a spatially restricted response, *e.g.*, at the place of injury, where immediate action of the cytokine might be necessary. In such cases, local activation is controlled by changes in the cell-cell and cell-ECM communication that lead to direct interactions between the L-TGF-β, structural proteins of the ECM, and components located at the cell surface of neighbouring cells. Proteolytic activity and mechanical forces will lead to L-TGF-β activation and cell signalling initiation. Importantly, local interference with L-TGF-β activation might be a safer therapeutic strategy than targeting the activated ligand, since it may tone down instead of totally blunting the effects of TGF-β.

Abbreviations

α-SMA, alpha-smooth muscle actin; BMDCs, bone marrow-derived dendritic cells; BMPs, bone morphogenetic proteins; cFn, cellular fibronectin; Co-Smad, co-mediator Smad; cRGD, cyclic arginine-glycine-aspartic acid peptide; ECM, extracellular matrix; ECM1, extracellular matrix protein 1; rECM1, recombinant ECM1 protein; HCC, hepatocellular carcinoma; HSCs, hepatic stellate cells; I-Smad, inhibitory Smad; LAP, latency-associated peptide; LLC, large latent complex; LTBP, latent TGF-β binding protein; L-TGF-β, latent transforming growth factor β; MMP, matrix metalloproteinase; PAI-1, plasminogen activator inhibitor-1; pFn, plasma fibronectin; RGD, arginine-glycine-aspartic acid; ROS, reactive oxygen species; R-Smad, receptor-regulated Smad; SLC, small latent complex; TGF-β, transforming growth factor β; TIMP-1, tissue inhibitor of metalloproteinase-1; TSP, thrombospondin.

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Conflict of interest

The authors declare that they have no conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

YL, FL, SW, and SD wrote the original draft. YL, WF, SW, and SD reviewed and edited the manuscript. SW and SD share the co-corresponding authorship.

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Supplementary data

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Author names in bold designate shared co-first authorship

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